The molecular machinery for lysosome biogenesis

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Summary
The lysosome serves as a site for delivery of materials targeted for removal from the eukaryotic cell. The mechanisms underlying the biogenesis of this organelle are currently the subject of renewed interest due to advances in our understanding of the protein sorting machinery. Genetic model systems such as yeast and *Drosophila* have been instrumental in identifying both protein and lipid components of this machinery. Importantly, many of these components, as well as the processes in which they are involved, are proving conserved in mammals. Other recently identified components, however, appear to be unique to higher eukaryotes.
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Introduction

Since the discovery of lysosomes in 1949, much has been learned about their structure, biochemical properties, composition and function. The mechanisms involved in their biogenesis, however, remain poorly understood. Over the past few years, there has been a virtual explosion in the characterization of the molecular machinery that directs lysosome biogenesis. Our growing understanding of the complex physical and functional relationships among components of this machinery is providing insights into the processes governing the generation and maintenance of this organelle. In this article, we review recent progress in the identification and functional analysis of components of the lysosome biogenesis machinery.

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Abbreviations: ALP, alkaline phosphatase; AP,adaptor protein; API, aminopeptidase I; ARF, ADP-ribosylation factor; Boss, Bride of Sevenless; *car*, *carnation*; CD-MPR, cation-dependent mannose 6-phosphate receptor; CI-MPR, cation-independent mannose 6-phosphate receptor; *cm*, *carmine*; CPY, carboxypeptidase Y; Cvt, cytoplasm to vacuole; *Dm*, *Drosophila melanogaster*; *dor*, *deep orange*; ECV, endosomal carrier vesicle; EEA1, human early endosome antigen protein 1; FYVE, Fab1 YGLO23 Vps27 EEA1; *g*, *garnet*; GGA, Golgi-localized y ear-containing ARF binding proteins; HPS1, Hermansky-Pudlak syndrome type 1; *lt*, *light*; MVB, multivesicular body; *or*, *orange*; PDGF, platelet derived growth factor; PI, phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; PI(3,5)P, phosphatidylinositol 3, 5-phosphate; PI-TP, phosphatidylinositol transfer protein; PM, plasma membrane; PVC, prevacuolar compartment; *rb*, *ruby*; *Sc*, *Saccharomyces cerevisiae*; SNARE, N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors; SNX, sorting nexin; TGN, *trans*-Golgi network; V, vacuole; vam, vacuolar morphology; VPS, vacuolar protein sorting

Characteristics of lysosomes

Lysosomes are membrane-bound organelles that serve as the major degradative compartment within the central vacuolar system of eukaryotic cells. In this role, lysosomes are the terminal destination for many endocytic, autophagic, and secretory materials targeted for destruction. Lysosomal degradation is critical to many physiological processes, including the turnover of normal cellular proteins, disposal of abnormal proteins, downregulation of surface receptors, release of endocytosed nutrients, inactivation of pathogenic organisms, and antigen processing. In addition, lysosomes play crucial roles in metal ion homeostasis and plasma membrane repair. Microscopic identification of lysosomes solely on appearance is difficult due to heterogeneity in morphology. Identification of lysosomes is aided by the presence of a number of key features including highly glycosylated integral membrane proteins, named lamps, limps, or lgps, found in the lysosomal single limiting membrane. Lysosomes also maintain an acidic lumen that houses numerous acid-dependent hydrolases. This broad spectrum of degradative enzymes affords complete digestion of materials delivered into lysosomes. In addition, lysosomes lack both the cation-dependent (CD) and cation-independent (CI) mannose 6-phosphate receptors (MPRs), which distinguishes them from late endosomal compartments. These hallmark features of lysosomes are shared with a family of so-called "lysosome-related organelles" that perform cell-specific functions unrelated to degradation.[1] This family includes lytic granules, platelet dense bodies, melanosomes, and insect pigment granules. Based on the similarities between lysosomes and lysosome-related organelles, they are thought to share common origins within the cell. The physiological importance of lysosomes and related organelles is evidenced by the myriad of diseases resulting from defects in their biogenesis or function.[1]

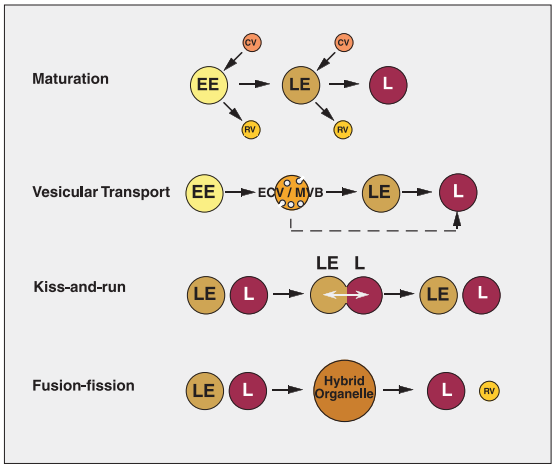

Figure 1. Models for lysosomal biogenesis requiring endosomal compartments. The maturation model suggests that early endosomes are formed by the coalescence of vesicles derived from the plasma membrane. These endosomes eventually mature to late endosomes and then to lysosomes through the addition of TGN-derived cargo vesicles and removal of materials in recycling vesicles destined for other compartments and/or the plasma membrane. The vesicular transport model predicts that early endosomes, late endosomes and lysosomes are distinct and stable structures. In this senario materials delivered to early endosomes are transported through an endosomal carrier vesicle, also described as the multivesicular body, to late endosomes which then mature into lysosomes. Transport from early endosomes directly to mature lysosomes through the intermediate compartment has also been proposed (denoted by hatched arrow). Models proposing interactions between late endosomes and lysosomes that contribute to lysosomal maturation include the kiss-and-run model. Here, the maintenance and, by extension, biogenesis of lysosomes occurs through a continuous cycle of late endosome-lysosome contact (or "kiss"), with an accompanying transfer of materials, followed by dissociation (or "run") (represented by double-headed arrow). The fusion–fission model is a variation of kiss-and-run in which late endosomes and lysosomes undergo heterotypic fusions producing a hybrid organelle containing markers of both compartments. Lysosome recovery (i.e. maturation) from the hybrid involves condensation of lysosomal contents and removal of endosomal materials through digestion or via a recycling vesicle(s). Abbreviations: EE, early endosome; LE, late endosome; L, lysosome; ECV, endosomal carrier vesicle; MVB, multivesicular body; CV, cargo vesicle; RV, recycling vesicle. See text for additional details and appropriate references.

Models for lysosome biogenesis

The question of how lysosomes form has captivated the interest of cell biologists for decades. Early microscopy studies suggested lysosomes form by direct budding from the Golgi complex. Later studies led to models proposing lysosome maturation from endosomal compartments (Fig. 1). The "maturation" model involves formation of early endosomes by coalescence of vesicles from the plasma membrane.[2] Removal of recycling vesicles and addition of *trans*-Golgi network (TGN)-derived vesicles converts these endosomes to late endosomes, and eventually to lysosomes. Alternatively, the "vesicle-transport" model postulates that early endosomes, late endosomes and lysosomes are stable compartments. Here transport proceeds from early endosomes through an endosomal carrier vesicle (ECV) with characteristics of a multivesicular body (MVB), to late endosomes which mature into lysosomes[1] or possibly to mature lysosomes directly.[2] Scenarios describing interactions between late endosomes and lysosomes include the "kiss and run" model. This model proposes that endosomes and lysosomes undergo repeated cycles of fusion and fission allowing transfer of materials and maintenance of mature lysosomes.[3] A recently proposed variation of "kiss and run" involves heterotypic fusion of late endosomes and lysosomes producing a hybrid organelle with subsequent lysosome reformation.[4] These models are not mutually exclusive and it is possible that cells employ more than one of these processes in lysosomal biogenesis. The fact that so many models have been proposed, however, reflects how poorly we understand these events.

Sorting pathways from the TGN to the lysosome

For any model of lysosomal biogenesis to work, a mechanism for selective sorting of materials bound for lysosomes must exist. Most newly synthesized lysosomal hydrolases are modified with mannose 6-phosphate and bind MPRs at the TGN. The hydrolase–receptor complexes then traffic in vesicles to either early or late endosomes.[5] Here, the hydrolases dissociate from the MPRs and are delivered to lysosomes, while the MPRs recycle back to the TGN. A second, and to date uncharacterized, pathway sorts lysosomal hydrolases to lysosomes in an MPR-independent fashion.[6] Like MPRs, lysosomal integral membrane proteins (e.g., lamp-1) can follow a similar pathway from the TGN to endosomes and lysosomes (i.e., the "direct" pathway). However, these proteins can also travel from the TGN to the plasma membrane, and subsequently to endosomes and lysosomes (i.e., the "indirect" or "salvage" pathway).[7] Different lysosomal membrane proteins appear to use each pathway to varying extents. The remainder of this discussion will focus primarily on the Golgi-to-lysosome sorting machinery and its contribution to the biogenesis of lysosomes and related organelles in lower eukaryotes and mammals.

Role of adaptor protein complexes in lysosomal sorting

The sorting of MPRs and lysosomal membrane proteins is mediated by signals present in the cytoplasmic domains of these proteins.[7] The signals are recognized by cytosolic molecules that are recruited onto membranes at sites of

vesicle formation (e.g., the TGN, plasma membrane and endosomes). Prime candidates for recognition molecules are the heterotetrameric adaptor protein (AP) complexes AP-1, AP-2, AP-3 and AP-4, all of which bind tyrosine-based and/or dileucine-based sorting signals.[8,9] It has long been assumed that AP-1 mediates transport of MPRs and lysosomal integral membrane proteins from the TGN to endosomes.[10,11] Genetic ablation of AP-1, however, has produced the unexpected findings that both CD-MPRs and CI-MPRs accumulate in endosomes rather than the TGN and that lysosomal membrane proteins are still transported to lysosomes.[12] This suggests that AP-1 plays a role in recycling MPRs from endosomes to the TGN. Two unrelated connector molecules, TIP47[13] and PACS-1,[14] have also been implicated in recycling of MPRs to the TGN. AP-2 is involved in rapid internalization from the plasma membrane and likely participates in endocytosis of MPRs and lysosomal membrane proteins trafficking via the indirect pathway to lysosomes. AP-3 has been implicated mainly in the biogenesis of lysosome-related organelles,[15,16] although it also appears to participate in sorting of some lysosomal membrane proteins to lysosomes from an intracellular site (i.e., the TGN or endosomes). Indeed, reduction in AP-3 levels or mutations in some of its subunits increase trafficking of lysosomal integral membrane proteins via the plasma membrane, although the steady-state localization of these proteins to lysosomes is only minimally affected.[16,17] AP-4 is localized to the TGN and could thus be involved in sorting to lysosomes, but there is currently no evidence for this function. A related coat protein complex, COPI, has also been shown to mediate sorting of some proteins between early and late endosomes.[18] In summary, AP complexes and COPI appear to participate in sorting en route to lysosomes, but it remains unclear which of these complexes is primarily responsible for targeting the bulk of lysosomal proteins to the lysosome itself. It is possible that the functions of some of these complexes are redundant, which might explain why abrogating expression of any one of them has only limited effects on the overall distribution of lysosomal proteins.

The GGA family of proteins are potential mediators of biosynthetic trafficking to lysosomes

Recent studies have identified a novel family of coat proteins, termed GGAs, which may be involved in biosynthetic protein transport from the TGN to the endosomal/lysosomal system.[19–23] This family comprises three members in mammals (GGA1, GGA2 and GGA3) and two members in yeast (Gga1p and Gga2p). All of these proteins have a modular structure consisting of an amino-terminal VHS (for Vps27p, Hrs, STAM homology) domain, a GAT (for GGA and TOM homology) domain, a variable linker region, and a carboxy-terminal GAE domain homologous to the ear domain of γ-adaptin (a subunit of AP-1). GGA proteins are not components of AP-1 and appear to exist as monomers. Immunofluorescence and immunoelectron microscopy have demonstrated that the mammalian GGAs localize to coated buds and vesicles in the area of the TGN. The recruitment of GGAs to the TGN appears to be mediated by members of the ADP-ribosylation factor (ARF) family of proteins, by virtue of a direct interaction between the GAT region of the GGAs and the GTP-bound form of ARFs.[19,20] The GAE domain of the mammalian GGAs, interacts with γ-synergin[23] a potential regulatory molecule that also binds the analogous domain of γ-adaptin. The linker regions of some of the GGAs have sequences that fit a consensus motif for binding to the scaffolding protein clathrin.[20] Disruption of both GGA genes in yeast results in impaired sorting of pro-carboxypeptidase Y (CPY) to the vacuole, the equivalent of the mammalian lysosome[20,21] suggesting that the yeast Ggaps are involved in vacuolar protein sorting. By analogy, the mammalian GGAs might be involved in biosynthetic protein transport to lysosomes.

Rabs and SNAREs involved in transport to lysosomes

Once carrier vesicles are formed and cargo proteins sorted into them, the vesicles need to be targeted and eventually fuse with acceptor compartments. A class of small GTP-binding proteins, termed rabs, are found at the plasma membrane and on organelles of the secretory and endocytic pathways and function in vesicle transport, and docking/fusion. In the GTP-bound state, rabs bind to membranes and recruit regulatory molecules and effectors of vesicle docking/fusion. Although the roles of the majority of rabs are unknown, Rab7 (an orthologue to yeast Ypt7p) and Rab9 have been localized to the surface of late endocytic compartments.[25] Rab7 appears to be required for transport to the lysosome[26] while Rab9 mediates MPR recycling from late endosomes to the TGN.[27] Expression of a constitutively active Rab7 mutant increases lysosomal fusion while dominant negative Rab7 mutants disperses lysosomes.[28] Earlier endosomal compartments are apparently unaffected by these treatments. These results suggest Rab7 is important for late endosome–lysosome fusion events.

Targeting and membrane fusion also require members of the vesicle-associated membrane protein (VAMP)/synaptobrevin, syntaxin, and synaptosomal-associated protein of 25 kDa (SNAP-25) families. These proteins are collectively known as N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors, or SNAREs, and are found on both vesicles (v-SNAREs) and target membranes (t-SNAREs).[29] Syntaxin 7 (an orthologue to yeast Pep12p) and VAMP-7, appear to mediate heterotypic and homotypic fusions involving lysosomes.[30] Syntaxin 8 is found on endosomes, lysosomes, and possibly the TGN and probably mediates sorting from early to late endosomes.[31] The degree

to which these, or other, mammalian SNARES are required for sorting to the mature lysosome itself remains uncertain. The functional interactions reported between the same v-SNAREs and t-SNAREs in multiple sorting pathways and between v-SNAREs or t-SNAREs with several partners suggests additional factors (such as Rab effectors and modified lipids, described below) are essential to sorting specificity. This view is supported by studies that demonstrate a promiscuity in SNARE interactions in vitro.[32] Recent in vitro studies of SNARE pairing in yeast, however, are in sharp contrast to this view.[33] Here, a systematic assessment of Golgi, vacuole and plasma membrane t-SNARE interactions with demonstrated or proposed v-SNARES showed a marked specificity in pairing.

Genetic studies in yeast provide insight into lysosomal biogenesis

Even though AP complexes, Rabs and SNAREs, and possibly the GGAs, play fundamental roles in transport to lysosomes, it is now clear that they represent only part of the lysosome biogenesis machinery. Many other components have been identified using genetic approaches with the yeast *Saccharomyces cerevisiae*. The elegant genetics developed for isolating mutants defective in protein sorting to the yeast vacuole has been invaluable in discerning conserved events in higher eukaryotes.

Routes for delivery of materials to the yeast vacuole

At least six pathways are involved in trafficking to the yeast vacuole: endocytosis from the cell surface, the cytoplasm to vacuole (Cvt) pathway, autophagy, vacuole inheritance during cell division, and two alternative biosynthetic pathways from the late Golgi complex referred to as the "carboxypeptidase Y (CPY) pathway" and the "alkaline phosphatase (ALP) pathway" (Fig. 2). Endocytosis is essential for regulating levels of membrane proteins such as the Ste2p and Ste3p pheromone receptors and requires a highly regulated interplay between a host of endocytic mediators and the actin cytoskeleton.[34] Proteins such as aminopeptidase I (API) and α-mannosidase I (Ams1p) are delivered from the cytoplasm to the vacuole through overlapping, non-classical vesicular pathways involving products of the CVT, AUT (autophagy) and APG (autophagocytosis) genes.[35] Vacuolar segregation into daughter cells of budding yeast requires products of the VAC (vacuolar partitioning) gene family, some of which also participate in vacuolar protein sorting.[36] Because of their direct relevance to lysosomal biogenesis, the CPY and ALP biosynthetic routes will be the focus of the remainder of this section.

The CPY sorting pathway

CPY is the prototype of a number of proteins (e.g. protease A, protease B, and subunits of the vacuolar ATPase) that traffic

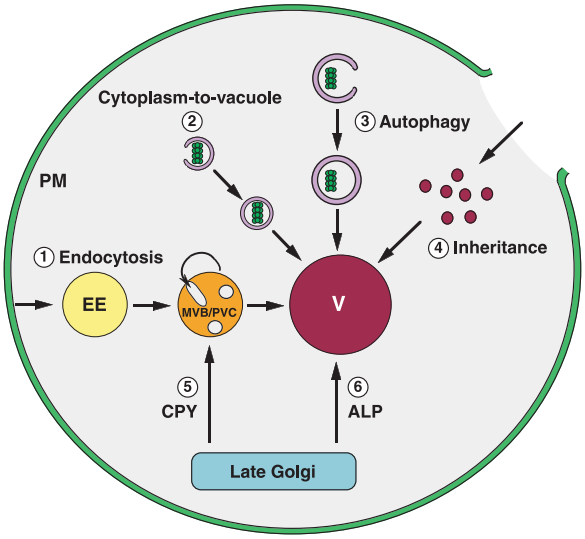

Figure 2. Schematic representation of the six pathways for delivery of materials to the yeast vacuole. (1) Endocytosis of materials (e.g., cell surface receptors targeted for destruction) from the plasma membrane is followed by trafficking to early endosomes then to late endosomes/MVBs (also manifested as the PVC in some sorting mutants). Here select cargo is internalized into luminal vesicles and other materials are recycled. Mature MVBs are then proposed to fuse with the lysosome for delivery of luminal vesicles/cargo which are turned over. (2) In the cytoplasm to vacuole (Cvt) pathway, cytoplasmic proteins such as oligomerized precursor aminopeptidase I (prAPI) (schematically represented in green) are internalized within a double membrane Cvt vesicle. Cvt vesicles then fuse with and deliver their material into the vacuole. (3) In autophagy, cytoplasmic proteins are enclosed in a double membrane autophagosome which fuses with the vacuole to release membranes and cargo for degradation. This pathway is similar to Cvt but is utilized primarily under starvation conditions. (4) Vacuolar Inheritance is initiated early in the cell cycle through the projection of a segregation structure from the vacuole toward the growing bud. This protrusion then vesiculates and vesicles travel into the bud (pictured) where they coalesce to form a vacuole for the daughter cell. (5) The CPY pathway sorts newly synthesized proteins from the late Golgi (the yeast equivalent to the mammalian TGN) to late endosomes/MVB/PVC. Materials are then delivered from the MVB to the vacuole as described above. (6) The ALP pathway functions as an alternative pathway to the CPY route but does not rely on a late endosomal/MVB/PVC intermediate compartment. Some cargo is sorted by the CPY pathway while other cargo selectively sorts via the ALP pathway. Abbreviations: PVC, prevacuolar compartment; MVB, multivesicular body; V, vacuole; PM, plasma membrane; EE, early endosome; CPY, carboxypeptidase Y; ALP, alkaline phosphatase. For additional details and appropriate references, see text.

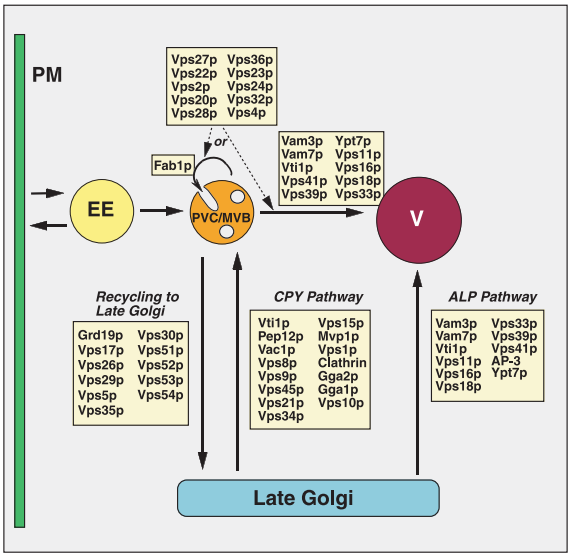

Figure 3. A schematic representation of the CPY and ALP biosynthetic sorting pathways from the late Golgi to the vacuole in yeast. Factors important for anterograde sorting from the late Golgi to the PVC/MVB and for MVB-to-vacuole sorting are listed. A number of factors are also shown whose functions appear to operate in MVB formation and/or transport from the MVB to the vacuole. Factors required for recycling from the MVB to the Golgi are indicated. Also shown are factors that are required for transport directly from the late Golgi to the vacuole via the ALP pathway. This list represents some of the better-known sorting proteins pertinent to our discussion and is not meant to be comprehensive. Many of these factors have multiple published names so the most commonly used terms are presented. Abbreviations: PVC, prevacuolar compartment; MVB, multivesicular body; V, vacuole; PM, plasma membrane. See text for detailed information concerning individual factors and appropriate references.

from the Golgi complex to the vacuole by way of a pre-vacuolar compartment (PVC) (Fig. 3). This pathway depends upon the function of over 50 VPS (vacuolar protein sorting) gene products, mutations in which result in CPY missorting to the periplasmic space and, often, abnormal vacuolar morphology. In a manner analogous to M6PR-mediated sorting in mammals, the CPY receptor, Vps10p, binds CPY at the Golgi complex via a targeting signal in the CPY amino-terminal propeptide. Sorting of the receptor–ligand complex to the PVC requires proteins involved in vesicle formation such as Gga1p, Gga2p[20,21] and clathrin[37] and the dynamin homologue Vps1p.[38] Other proteins are involved in targeting to and fusion with the PVC, including Vps21p (an orthologue to the mammalian Rab5 GTPase; see Ref. 39), Vps9p (a putative guanine nucleotide exchange factor; Ref. 40), Vac1p/Vps19p,[41] Vps45p (a member of the SEC1 family implicated in general secretion; Ref. 42), Pep12p/Vps6p (a t-SNARE; Ref. 43); Vti1p (a v-SNARE; Ref. 44); Sec18p (a NSF orthologue; Ref. 45). The Vps15p and Vps34p kinases contribute to this sorting step through the phosphorylation of specific Golgi/endosome lipids, which subsequently recruit effector proteins (discussed below; see Ref. 46).

At the PVC, Vps10p dissociates from CPY and recycles to the Golgi complex in a process requiring Vps29p, Vps26p, Vps35p, Vps5p and Vps17p.[47] These proteins associate into a large membrane-associated complex, referred to as the "retromer", which consists of two subcomplexes containing Vps29p/Vps26p/Vps35p and Vps5p/Vps17p. A model for retromer function in Golgi retrieval has been proposed by Seaman et al. which suggests that Vps29p/Vps26p/Vps35p act in selection of cargo (*e.g.* Vps10p) while Vps5p/Vps17p drive vesicle budding from the PVC membrane. Mutants deficient in these factors mislocalize Vps10p to the vacuole and secrete CPY, probably because Vps10p becomes limiting at the Golgi complex.[47] A function for Vps30p in retrograde transport has been suggested but remains unclear. Interestingly, Vps30p, like its putative mammalian orthologue *beclin 1*, has also been implicated in autophagy.[48,49] Recycling from the PVC to the Golgi also requires the Vps52p–Vps53p–Vps54p complex, which peripherally associates with the late Golgi membrane.[50] *vps52, vps53*, and *vps54* mutants, like mutants in the retromer complex, display Vps10p missorting to the vacuole and CPY secretion. This phenotype and the localization to the late Golgi suggest a role for this complex in vesicle docking/fusion.

Another set of Vps proteins appears to be required for vesicular formation/transport at the PVC. This proposed function is based on observations that mutants such as *vps27* and *vps28* accumulate exaggerated PVCs containing vacuolar hydrolases such as CPY and proteinase A and the endocytic marker Ste3p.[51,52] *vps27* and *vps28* mutants also concentrate Vps10p and other late-Golgi proteins in the PVC. Vps4p.[53] is a member of the AAA-type family of ATPases implicated in a number of diverse cellular processes. Vps4p binds to PVCs in an ATP-dependent manner where it potentially regulates interactions between other proteins acting at this step.[53] It appears possible these proteins are involved in the formation and/or maturation of MVBs. The exaggerated PVC observed in these mutants is believed to represent an altered from of the MVB in which vesicle budding into the lumen is inhibited.[54] This defect appears to block delivery of proteins (such as cell-surface receptors targeted for degradation) from the MVB to the vacuole lumen, which can occur through MVB–vacuole fusion.

Finally, fusion of prevacuolar intermediates with the vacuole requires another set of proteins including Ypt7 (an orthologue to the mammalian Rab7 GTPase), Vti1p (a v-SNARE), Vam3p (a t-SNARE), Vam7p (a SNAP-25 homologue), a complex composed of Vps18p, Vps11p, Vps16p, and

Vps33p and another complex containing Vps41p and Vps39p.[55,56] The latter proteins appear to be required not only in the CPY pathway but also in the ALP pathway .[57]

The ALP sorting pathway

In contrast to CPY, ALP and the t-SNARE Vam3p travel from the Golgi complex to the vacuole independently of the pre-vacuolar compartment. Sorting in this pathway is mediated instead by the yeast homologue of mammalian AP-3 and by Vps41p–Vam2p.[57,58] (Fig. 3). Selective diversion into this pathway at the Golgi complex requires specific sorting motifs in the ALP and Vam3p cytoplasmic tails.[58,59] Recently, the interplay between AP-3 and Vps41p has come under closer examination. Using a *vam3*^{ts} mutant, Rehling et al.[60] induced accumulation of membrane vesicles containing ALP, Vam3p, and AP-3. Formation of these vesicles at the Golgi complex was demonstrated to be dependent on AP-3. While binding of AP-3 to membranes was not obviously influenced by Vps41p, Vps41p did associate with AP-3 through its δ-adaptin subunit. This suggests possible roles for Vps41p in recruiting factors needed for AP-3 vesicle formation or as a scaffold for these vesicles. One possible candidate for recruitment may be Vps39p, which forms a complex with Vps41p.[56] and, like Vps41p, is encoded by a gene identified in screens for mutants affecting the maintenance of vacuole morphology (*vam*). Furthermore, *vps39* mutants display a severe ALP processing defect similar to *vps41* mutants.[56] There is now evidence that Vps41p (and other proteins including Vps39p) may be important not only in the generation of vesicles but also in allowing vesicle docking at the vacuole. The Vps41p–Vps39p complex, for example, has been shown to mediate docking prior to vacuole homotypic fusions[61] and absence of Vps39p or Vps41p results in vacuole fragmentation.[56] The function of Vps41p and Vps39p in vesicular transport is probably not specific to the ALP pathway, however, as *vps41* and *vps39* mutants show substantial missorting of a number of vacuolar hydrolases including CPY (Refs. 56,62). Clathrin, Gga1p and Gga2p are apparently not required for sorting ALP to the vacuole. Interestingly, while AP-3 mutants display missorting of ALP and Vamp3, the yeast homologues of the AP-1 and AP-2 complexes do not appear critical for sorting to the vacuole or endocytosis. Functional redundancy alone can not explain this, as elimination of all AP complexes in yeast does not result in a synthetic sorting defect (63).

The yeast vacuolar sorting machinery is conserved in higher eukaryotes

A survey of sequence databases reveals that many proteins involved in vacuolar sorting and biogenesis in yeast have orthologues in higher eukaryotes such as the fruit fly *Drosophila melanogaster* and mammals (for examples see Table 1). Such a high degree of conservation for these proteins suggests that the basic molecular mechanisms in which they are involved are also similar. Indeed, whenever Vps orthologues in higher eukaryotes have been analyzed for function, they have been found to act similarly to their yeast counterparts.

The conservation of the lysosomal/vacuolar sorting machinery has been especially evident in studies of the relationship between lysosomes and lysosome-related organelles. Pigmentation mutants in *Drosophila* have been found to bear mutations in genes encoding orthologues of yeast proteins involved in both the CPY and ALP pathways. Defective expression of the gene encoding the δ-adaptin subunit of *Drosophila* AP-3 was initially associated with defective pigment granule biogenesis in *garnet* (*g*) mutant flies.[64] Since then, defects in the other AP-3 subunits (σ3, μ3, and β3) have been linked to reductions of pigment granules in the *orange* (*or*), *carmine* (*cm*), and *ruby* (*rb*) mutants, respectively.[65–67] Moreover, mutations in AP-3 subunits in the *mocha*[15] and *pearl*[68] mouse strains and in human patients with Hermansky-Pudlak syndrome type 2,[16] cause defects in melanosomes (mammalian pigment granules), platelet dense bodies, and lysosomes from reticulo-endothelial cells. Taken together, these observations indicate a role for the AP-3 complex in the biogenesis of lysosomes and lysosome-related organelles in higher eukaryotes. Given the ability of the AP-3 complex to recognize sorting signals, the organellar defects observed in AP-3-deficient animals are probably due to impaired sorting of enzymes, transporters or structural proteins to those organelles. This is consistent with the role of yeast AP-3 in sorting ALP and Vam3p to the vacuole.

The *g*, *or*, *cm*, and *rb Drosophila* strains belong to a family of eye color mutants termed the "granule group". This family also includes the mutants *deep orange* (*dor*), *carnation* (*car*), and *light* (*lt*), which contain defects in *Drosophila* orthologues of yeast Vps18p, Vps33p, and Vps41p, respectively.[69–71] Moreover, genetic interactions occur between granule group mutants implying an involvement for their gene products in similar molecular events. While pigmentation phenotypes are the most visible effect of mutations in these pathways, defects in trafficking to lysosomes similar to those in yeast *vps18* and *vps33* mutants are also observed in *dor* and *car* flies.[70]

Studies of late endocytic trafficking using a Bride of Sevenless (Boss)-HRP transgene have shown that mutations in *dor* inhibit trafficking to lysosomes.[70] In the *dor* mutant, internalized Boss accumulates above normal levels most likely as a result of defective transport of Boss and/or lysosomal hydrolases to maturing lysosomes. Furthermore, giant MVBs containing unusual internal vesicles are observed in *dor* cells. This may indicate inhibited MVB maturation into lysosomes. The lysosomal sorting defect and occurrence of large MVBs in the *dor* mutant and the established role of yeast Vps18p and Vps33p [55] in multivesicular body–vacuole fusion and vesicle docking, respectively, strongly support a conserved function for the Dor–Car complex in *Drosophila*.

Table 1. Some conserved factors involved in the biogenesis of lysosomes/vacuoles[1]

Mammalian[1]	Orthologues[2]	Function(s)[3]
σ3	*Sc* Aps3p; *D* Orange	AP-3 subunit; sorting to the lysosome/vacuole and related organelles
μ3	*Sc* Apm3p; *Dm* Carmine	AP-3 subunit; sorting to the lysosome/vacuole and related organelles
β-3/Pearl	*Sc*Ap16p; *Dm* Ruby	AP-3 subunit; sorting to the lysosome/vacuole and related organelles
δ-adaptin/mocha	*Sc* Ap15p; *Dm* Garnet	AP-3 subunit; sorting to the lysosome/vacuole and related organelles
GGA1, GGA2, GGA3	*Sc* Gga1p, Gga2p	Putative ARF1 effectors; sorting to the vacuole
Rab7	*Sc* Ypt7p	GTP-binding protein; sorting to lysosome (late endosome)/vacuole
Rab5	*Sc* Vps21p	GTP-binding protein; sorting to endosomes
Syntaxin 7	*Sc* Pep12p (Vps6p)	t-SNARE; mediates membrane fusion events
hVPS4/SKD1	*Sc* Vps4p	AAA-type ATPase; endosomal sorting
VPS34	*Sc* Vps34p	PI-specific 3-kinase; sorting to endosomes and the lysosome/vacuole
VPS15 (p150)	*Sc* VPs15p	Membrane-associated kinase; stimulates VPS34/VPs34p; sorting to endosomes and the lysosome/vacuole
EEA1	*Sc* Vac1p	FYVE-protein; PI3P effector; mediates membrane fusion events
Hrs	*Sc* Vps27p	FYVE-protein; PI3P effector; endosomal sorting

[1]This list represents some of the move well-studied sorting factors with demonstrated sequence and functional conservation between mammals (human/mouse) and *Saccharomyces cerevisiae* (*Sc*) and/or *Drosophila melanogaster* (*Dm*) and is not meant to be completely comprehensive. Factors presented appear involved primarily in biosynthetic sorting pathways to the lysosome/vacuole.
[2]Many proteins have multiple published names so the more commonlty used terms and presented. In some cases a second common name is given in parenthesis.
[3]The function(s) listed represent the generally perscribed function(s) and/or function(s) most relevant to this study. Refer to text for additional details and references.

The mammalian VPS4 and SKD1 proteins, like the related yeast class E protein Vps4p,[53] are members of the AAA-type family of ATPases.[72,73] Overexpression of dominant-negative (i.e. ATPase-defective) mutant forms of mammalian VPS4 or SKD1 inhibits vacuolar sorting and leads to the accumulation of abnormal vacuolar-like compartments.[72,73] These structures appear similar to the PVCs seen in *vps4* mutant cells. Together, these findings suggest similar roles for yeast Vps4p and human VPS4 and mouse SKD1 in vesicle formation/transport at endosomes and/or the PVC (MVB).

Mammalian sorting nexin 1 (SNX1), originally identified in screens for proteins interacting with the epidermal growth factor receptor,[74] and SNX2 are putative orthologues[75] to yeast Vps5p, a subunit of the retromer.[47] SNX1 and SNX2 associate with human orthologues to yeast Vps26p, Vps29p, and Vps35p[76] suggesting the presence of a mammalian equivalent to the yeast retromer. A third sorting nexin, SNX3, is an apparent orthologue to yeast Grd19p.[76] Grd19p is implicated in the retrieval of certain late-Golgi proteins (e.g. Kex2) from the PVC via interactions with sorting motifs but is not required for recycling of Vps10p or for vacuolar sorting.[77] Another SNX-related protein in yeast, Mvp1p, interacts with Vps1p (the yeast homologue to dynamin) at the Golgi to promote sorting to the vacuole.[78] Besides displaying overall sequence homology, the SNX proteins and their yeast orthologues contain a conserved $\sim$100 residue PX domain possibly involved in protein–protein interactions.[75] These findings suggest the mammalian SNX family participates in late-Golgi retrieval and lysosomal sorting pathways similar to those operating in yeast. However, further functional characterization is needed to confirm this.

Phosphoinositides as mediators of protein sorting to the yeast vacuole and mammalian lysosome

So far we have focused our discussion on proteins involved in lysosome biogenesis. However, lipids also play important, though less well understood, roles in this process. Phosphatidylinositols (PIs) are lipids that apparently function as second messengers in a variety of trafficking events in yeast as well as mammals.[79] Phosphorylation of PI to PI3P by the PI-specific 3-kinase Vps34p is required for vacuole sorting through the CPY pathway. Vps34p is recruited to Golgi and endosomal membranes and stimulated to phosphorylate by the membrane-associated kinase, Vps15p.[46] Inhibition of PI phosphorylation in *vps34* and *vps15* mutants produces sorting defects as well as abnormal vacuolar morphology,[76] illustrating the importance of PI3P-dependent sorting to vacuole biogenesis. But how does PI3P regulate these processes? A likely explanation comes from findings implicating PI3P in recruiting and/or activating FYVE-containing effectors of vesicular trafficking. The FYVE (for Fab1, YGLO23, Vps27, and EEA1) domain is similar in structure to Zn^{2+} RING-finger motifs[80] but binds specifically to PI3P.[81] In Golgi to endosome sorting, PI3P recruits FYVE-containing protein Vac1p/Vps19p (an orthologue to mammalian EEA1), which

interacts with other effectors including Vps45p and activated Vps21p (yeast Rab5) .[82,83] In this way, Vac1p may act as an adaptor for transport factors mediating vesicle docking and fusion.[83] PI3P also interacts with Vps27p (an orthologue to mammalian Hrs), a FYVE-containing factor probably required for endosome maturation.[84] Another FYVE-containing protein Fab1p mediates vacuolar biogenesis through phosphorylation of PI3P to PI(3,5)P at endosomes. Fab1p, and PI(3,5)P, are apparently not required for sorting through the CPY or ALP pathways but for maintenance of vacuole size and morphology and generation of multivesicular bodies.[54,85] A role similar to PI3P in recruiting cytosolic factors is likely to be revealed for PI(3,5)P. As *fab1* has been isolated in screens based on defective vacuolar partitioning into daughter cells, it seems that components of cell cycle-dependent membrane fission machinery may be prime candidates for PI(3,5)P binding.

The mechanisms of PI regulation of trafficking in mammals appear similar to those originally described in yeast. Human orthologues to both yeast Vps34p and Vps15p have been identified. hVPS34, like its yeast counterpart, is specific for the production of PI3P from PI.[86,87] and associates with human VPS15, termed p150, in vivo.[86,87] This association stimulates hVPS34 kinase activity approximately three fold, similar to the Vps15p activation of Vps34p to phosphorylate PI to PI3P at the yeast Golgi. In addition, phosphatidylinositol transfer protein (PI-TP) (an orthologue to yeast Sec14p), a factor implicated in regulating other mammalian PI kinases.[88] physically associates with this complex and further stimulates production of PI3P in in vitro kinase assays.[87] Addition of the fungal chemical wortmannin, which inhibits mammalian PI3P kinases, reduces sorting of Cathepsin D as well as endocytosed platelet-derived growth factor (PDGF) receptors to the lysosome.[89,90] In addition, microinjection of anti-hVPS34 antibodies similarly reduces post-endocytic sorting of PDGF receptors and transferrin.[91] These data thus show a strong link between hVPS34/p150p-dependent generation of PI3P and sorting to lysosomes. As in yeast, PI3P lipids in mammalian cells probably act as second messengers through recruitment of FYVE-containing effector proteins. One such effector is the human early endosome antigen (EEA1) protein (yeast Vac1p/Vps19p) which, in association with Rab5 (yeast Vps21p), is believed to mediate membrane heterotypic fusions involving endosomes.[92] EEA1 associates with PI3P on endosomes through its FYVE domain.[81] This interaction is inhibited by wortmannin and anti-hVPS34 antibodies.[91] A second effector may be the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) protein (yeast Vps27p) which also localizes to endosomes in a manner dependent on its FYVE domain and PI3P.[93] The findings that Hrs localization to endosomes is inhibited by wortmannin and cells from Hrs knockout mice contain enlarged early endosomes are further support for a role for Hrs in PI3P-mediated sorting.[94] Recent studies using a FYVE probe have localized PI3P to early endosomes and intraluminal vesicles of MVBs in mammals and yeast, but not to late endosomal compartments.[95] These findings further underscore the conserved role of PI3P as well as suggesting a possible function in maturation of early endosomes to MVBs.

Genes involved in lysosome biogenesis that have no orthologues in yeast

Higher eukaryotes are obviously more complex than yeast and it is thus not surprising that the biogenesis of lysosomes and related organelles requires additional genes. Mammals often have several isoforms of VPS genes that are single-copy in yeast, suggesting greater redundancy or diversification of function. Some of the higher eukaryotic VPS orthologues display changes in the domain organization of the protein products relative to yeast, reflecting adaptive changes during evolution. In addition, lysosome biogenesis in higher eukaryotes requires certain genes that have no homologues in yeast. An example is the *Drosophila* Hook gene, which encodes a cytoplasmic protein localized to endocytic compartments. *hook* mutant flies display a dramatic reduction in the number of multivesicular bodies and an increase in the number of multilamellar lysosomes.[96] These findings imply a role for Hook in lysosomal biogenesis possibly through positively regulating MVB–lysosome fusion or transport of internalized ligands to maturing lysosomes. Supporting this latter notion is the observation that several classes of receptors are degraded at an increased rate in the *hook* mutants possibly due to accelerated lysosomal delivery.[97]

Analysis of pigmentation defects in mice and humans have also uncovered novel genes that are involved in the biogenesis of lysosome-related organelles. The most obvious manifestation of mutations in these genes is reduced pigmentation due to abnormal melanosomes. Other lysosome-related organelles such as platelet dense bodies are also abnormal, however, and lysosomes from reticulo-endothelial cells display accumulation of undegraded materials. This suggests that these genes participate in pathways that are common to lysosome-related organelles and at least a subset of lysosomes. The *pale ear* mutant mouse and human patients with Hermansky-Pudlak syndrome type 1, for example, have been found to bear mutations in a cytosolic protein known as HPS1p.[1] An orthologue of this protein exists in *Drosophila* but not in yeast. Unfortunately, this protein does not resemble any protein of known function, nor does its amino acid sequence suggest the presence of described functional domains. The *pallid* mutant mouse bears a mutation in a gene encoding a novel protein known as pallidin that has no apparent orthologues in either *Drosophila* or yeast. Other than its sequence, the only feature known about this protein is that it interacts with syntaxin 13, a t-SNARE.[98] It is likely that cloning of other genes that are mutated in mouse pig-

mentation mutants and patients with Hermansky-Pudlak syndrome will result in the identification of additional components of the lysosome biogenesis machinery that are specific to metazoans.

Concluding remarks

Biochemical and genetic analyses of lysosomal targeting have produced an extensive catalog of proteins involved in various aspects of lysosome biogenesis. This catalog is likely to expand considerably in the next few years as new components of the machinery are identified using evolving experimental tools, including in vitro transport assays, novel genetic screens and whole genome sequencing. Although this machinery may seem overwhelmingly complex at present, it is likely to appear even more complex in the coming years. The assignment of specific biochemical functions to components of the machinery, the establishment of physical and/or functional relationships between them, and the placement of the processes in which they are involved in a cellular context, however, will undoubtedly result in a new level of understanding of lysosome biogenesis. The mechanistic insights derived from these studies might then resolve some of the long-standing controversies about how lysosomes form and function.

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